

Laboratory rearing of sardine larvae, *Sardina pilchardus* (Walb.), and early effects of starvation: a preliminary experiment

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ABSTRACT

A rearing experiment of larval sardine is described. Preliminary results on the early effects of starvation on larval growth, survival and digestive system histology are also presented.

Fed larvae showed a normal morphological development under laboratory conditions. Observed growth and mortality rates are comparable to the values for other Clupeoid larvae cited in the literature.

Our results suggest that growth is depressed and mortality increases soon after the onset of starvation conditions. No signs of degenerative changes in digestive organs were observed. However, larvae subjected to food deprivation could be distinguished from fed larvae by the lack of histological evidence of digestive activity.

Key words: *Sardina pilchardus*, larval stages, rearing, growth, development, mortality, histology.

RESUMEN

Cultivo en laboratorio de larvas de sardina, *Sardina pilchardus* (Walb.), y efectos iniciales de inanición: una experiencia preliminar.

En este trabajo se describe una experiencia de cultivo de larvas de sardina, *Sardina pilchardus*, presentándose los resultados preliminares de los primeros efectos de inanición en el crecimiento, supervivencia y en la histología del sistema digestivo de las larvas.

Las larvas alimentadas mostraron un desarrollo morfológico normal en condiciones de cultivo. Los datos observados de crecimiento y mortalidad fueron comparables a los valores que están citados en la bibliografía para larvas de clupeidos. Nuestros resultados sugieren que el crecimiento disminuye y la mortalidad aumenta tan pronto como se producen condiciones de inanición, no observándose cambios de degeneración en los órganos digestivos. Sin embargo, las larvas a las que no se les suministro comida pudieron diferenciarse de las larvas alimentadas al no encontrarse en los cortes histológicos evidencia de actividad digestiva.

Palabras clave: *Sardina pilchardus*, estados larvarios, cultivo, crecimiento, desarrollo, mortalidad, histología.

INTRODUCTION

Sardine stocks from the Atlantic Coast of the Iberian Peninsula support important fishing activities of both Portuguese and Spanish fleets. The annual recruitment to these stocks shows a high variability which may be reflected in overall stock abundance, thus affecting the fishery.

Studies on the factors that influence recruitment variability of this species are in progress. Starvation, predation and advection are some of the factors that might affect the success of a year-class (Lasker, 1981). Starvation may be one of the most important mortality factors for the larval stage, and consequently one of the determinants of recruitment (O'Connell, 1980; Theilacker, 1986; Setzler-Hamilton *et al.*, 1987).

Knowledge about the early life history of sardine, *Sardina pilchardus* (Walb.), has been based almost exclusively on the collection of samples at sea (Karlovac, 1967; Southward and Demir, 1974; Ré, 1986). The establishment of rearing techniques that will enable us, in the near future, to develop experiments on the resistance of these species to starvation is of the utmost importance.

This paper describes an experiment to rear sardine larvae from sea-collected eggs, and presents information on the early effects of starvation. The only previous attempt to rear sardine larvae was reported by Blaxter (1969). Recently, Olmedo *et al.* (1990) succeeded in inducing spawning through hormone injection on a captive sardine stock. Eggs spawned were used to study the effect of temperature on the development of egg and yolk-sac larval stages (Miranda, Cal and Iglesias, 1990).

In the present study, one batch of larvae was fed, and another two batches were deprived of food throughout the experimental period. Results on survival, morphological development, growth and digestive system histology of fed and unfed larvae are presented. We regard these as preliminary results, since the rearing techniques employed, in particular rearing container size, are not optimal for larval rearing. Furthermore, the experimental period was not long enough for an accurate assessment of the effects of starvation on sardine larvae.

MATERIAL AND METHODS

Egg collection

Sardine eggs were collected off the northern Portuguese coast on 13 January 1989. The experiment was carried out at the Aquaculture Laboratory of the Instituto Español de Oceanografía in Vigo, Spain.

Plankton samples were obtained from oblique and horizontal Bongo net tows (net speed: 50 m/min) at the upper 20 m of the water column. They were stored in 10 l capacity plastic bags (1/3 water, 2/3 air) and placed in insulated boxes during the transport to the laboratory. Sardine eggs were separated with pipettes from these samples, but no attempt was made to count or to sort them by developmental stages.

An estimate of the number of eggs was computed from the numbers of hatched larvae and of dead eggs removed from the incubation container.

Rearing techniques

About one thousand eggs were placed in the incubation containers. These consisted of four plastic beakers (5 l) with transparent walls. They were filled with 1 μ m filtered sea water and immersed in a circulating water bath ($T=14.5^{\circ}\text{C}\pm 0.2^{\circ}\text{C}$) inside cylindrical-conical tanks (100 l) with black walls and white bottoms.

Hatching took place in the three days following the introduction of the eggs in the incubation containers. However, only on the second day was the number of larvae sufficient for further study.

Recently hatched larvae were transferred with pipettes from the incubation containers to smaller ones (2 l), which were immersed in the same water baths. Larvae were distributed into three rearing containers: container *F* (Fed) with 250 larvae, container *U*₁ (Unfed 1) with 150 larvae and container *U*₂ (Unfed 2) with 100 larvae.

Temperature of the larval rearing containers ranged from 14.6 $^{\circ}\text{C}$ to 16.1 $^{\circ}\text{C}$. Mean values for salinity, pH and dissolved oxygen were, respectively, 34, 8.05 and 8.9 mg/l. Gentle aeration was provided. The experiment was terminated when all the larvae were dead.

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Larvae from container *F* were fed on *Brachionus plicatilis*, enriched for 24 hours with the unicellular algae *Isochrysis galbana*. Food was added once a day, starting five days after hatching. Food density was monitored every day, and whenever necessary, additional food was provided to keep an average concentration of 20 rotifers/ml. No food was offered to the larvae in containers *U*₁ and *U*₂.

Every day, before the control of food density, about 20% of the water in each container was replaced. Materials deposited on the container bottoms were siphoned, and dead larvae collected and counted for the estimation of survival rates.

Sampling

Samples of 8 to 11 larvae were taken each day. During larval yolk-sac stage, only one daily sample with larvae from the three containers was taken. Thereafter, samples were taken each day from containers *F* and *U*₁. Larvae from container *U*₂ were not sampled due to their low initial number.

Half of the sampled larvae were preserved in 4% buffered formal-saline and the other half were preserved in 3% glutaraldehyde for histological examination (see **Histological Techniques**).

Gut contents were analyzed by transparency. Occasionally, it was possible to dissect the posterior region of the gut and remove food particles. Rotifer eggs and loricae were often removed intact and measured.

Survival

On the first day after hatching, dead larvae were removed from the egg incubation containers. So, a single percentage for larval survival was computed on this day. On the following days, dead larvae were collected from the larval rearing containers (*F*, *U*₁ and *U*₂), giving separate estimates of larval survival. We assumed that the first of these estimates corresponds to day 2 of the experiment.

At the end of the experiment, we observed a discrepancy between the initial number of larvae placed in each container

and the total numbers of dead larvae. This discrepancy suggests that not all dead larvae were detected during the daily removal operation. For the correction of daily mortality values, the number of missing larvae was added to the total number of dead larvae in each container, and a similar daily distribution of the two values was assumed. The correct number of dead larvae on day *i*, *d*_{*i*}, was computed as:

$$d_i = d_{o_i} + M \frac{d_{o_i}}{D}$$

where *d*_{*i*} = number of observed dead larvae on day *i*, *M* = number of missing larvae at the end of the experiment and *D* = total number of observed dead larvae during the experiment.

Larval survival rates, *S*_{*n*}, were computed as the percentage of the initial population on each container surviving through day *n*:

$$S_n = \left[1 - \frac{\sum_1^n d_i}{P} \right] \times 100$$

where *P* = initial number of larvae.

Growth

Standard lengths of all sampled larvae were measured one week after preservation. No correction factor for shrinkage was employed. However, according to the literature, it is possible to make a correction for standard length. The ratio (laboratory preserved size divided by previous live size) averaged for standard length of northern anchovy (*Engraulis mordax*) was 0.92 after shrinkage in formalin (Theilacker, 1980). Because hatching was monitored at 24-hour intervals, larvae sampled on the first day of the experiment could be from 0 to 24 hours old. So, we assumed that larvae were, on average, 0.5 days old.

Means and 95% confidence limits for larval standard length were computed. The sample from day 8 (age = 7.5 days) was not included in growth analysis because it had only three larvae.

Length and age data were fit to a Laird-Gompertz growth model (Zweifel and Lasker, 1976).

Histological Techniques

Larvae were preserved in 3% glutaraldehyde in 0.2 M phosphate buffer. Three hours after preservation, the fixing solution was replaced by three changes of 0.2 M phosphate buffer, at half hour intervals. Dehydration was carried out in a gradual ethyl alcohol series.

Larvae were embedded in glycol-methacrylate (2-hydroxy-ethyl-methacrylate) (Bennett *et al.*, 1976) and sectioned at 1-2 μm in the sagittal plane with a "Ralph" type glass knife (Lindner and Richards, 1978).

Sections were stained in 0.4% Toluidine Blue. The Periodic-Acid-Schiff reaction was used to demonstrate glucosidic substances. Glycogen deposits were identified through diastase control. Harris hematoxylin was used as a counterstain.

RESULTS

Morphological development

Newly hatched larvae had a long slender body with a well-developed finfold. The head was round, bending over the yolk-sac. The eyes were still unpigmented. At this stage, the neuromasts (one pair on the head and eight pairs laterally on the trunk) were probably the only functional sense organs. Two elliptical otic capsules were visible. The primordials of the caudal fin rays began to differentiate. The alimentary canal was a long straight tube, still closed at the extremities. It narrowed at the end to form a thin rectal canal.

One day later, the head straightened, becoming almost free from the yolk-sac membrane. The pectoral fins began to differentiate, and the pelvic fins developed a fan-shaped form. A slight invagination at the ventral surface of the head indicated the development of the mouth.

Jaw development and eye pigmentation began at 3.5 days. At this time, the insertion of the pectorals become oblique and fin rays appeared, the otic capsules enlarged and the branchial apparatus began its development.

Eye pigmentation, the development of a functional mouth and the opening of the anus were complete 5.5 days after hatching.

Growth and development of the organs differentiated during the yolk-sac stage proceeded, but no major changes in larval morphology were observed until 9.5 days of age. No morphological differences were found between feeding and non-feeding larvae.

Behavior and feeding

After hatching, sardine larvae spent most of the time motionless, floating upside down. Passive drifting was occasionally interrupted by sudden and short periods of swimming activity. Avoidance reactions at the approach of a pipette were already observed at this stage.

Larvae gradually became more active, moving throughout the water column in a horizontal position. However, even after the addition of food, they tended to remain near the container bottoms, showing no reaction to the light. This unusual behavior may have been due to the experimental conditions. In fact, larvae may have been attracted by the white color of the tank bottoms where the rearing containers were immersed.

Larvae began feeding five days after hatching. They ingested rotifer eggs and adults with 87.5 μm to 116.4 μm width. Most larvae also had phytoplankton cells in their guts. Food residues were always observed at the posterior half of the midgut and at the hindgut.

It was not possible to establish the relationship between larval age and prey size, but we observed that 9.5-day-old larvae were able to ingest food particles up to 160 μm width.

The typical feeding behavior of Clupeoid larvae (S-striking posture) (Blaxter and Hunter, 1982) was observed.

Digestive system histology

The histomorphology of the digestive system of post yolk-sac sardine larvae was described by Silva (1990). Here, we will refer to the development of this system from hatching to first-feeding stage, and point out some of the effects of food deprivation.

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At hatching, the gut wall was composed of a mucosa of simple cuboidal epithelium and a thin layer of fibrous connective tissue. The epithelial cells were higher in the posterior half of the gut. A narrow lumen was open throughout the length of the gut, but was partially occluded in the anterior region by pressure from the yolk-sac. The liver and the pancreas lay dorsal to the posterior end of the yolk mass. Both were elongate compact organs, composed of large polygonal cells. It was often difficult to distinguish them in sagittal sections because of their morphological and histological resemblance.

In 2.5-day-old larvae, the pharyngic port began to differentiate, separating the bucco-pharynx from the rest of the gut. The buccal cavity was lined by a simple squamous epithelium. The cartilage of the developing jaws and branchial arches showed a high degree of cellular organization, although cartilaginous matrix was still absent. At the pharyngic port, the mucosa comprised several layers of squamous epithelium. Mucous

cells were not yet apparent. Posterior to this region, the structure of the gut wall remained unchanged.

At this stage of development, the liver and the pancreas were clearly distinguished. The liver was composed of two or three layers of large, irregularly arranged, polyedric cells, containing numerous small clear vacuoles. In the pancreas, the development of an acinar structure was evident. Acinar cells were pyramidal, with basal nuclei and strongly basophilic cytoplasm. Apically, small zymogen granules were visible. An incipient gall bladder lay between the anterior ends of the two glands.

Two days later, numerous mucous cells had already appeared at the pharyngic port (fig. 1). A muscularis of striated fibers began to differentiate at the foregut. About midway along the length of the gut, a slight constriction indicated the pyloric port which separates the foregut from the midgut. The epithelial cells of the midgut then presented a cylindrical shape, with a well-developed striated border and numerous small clear

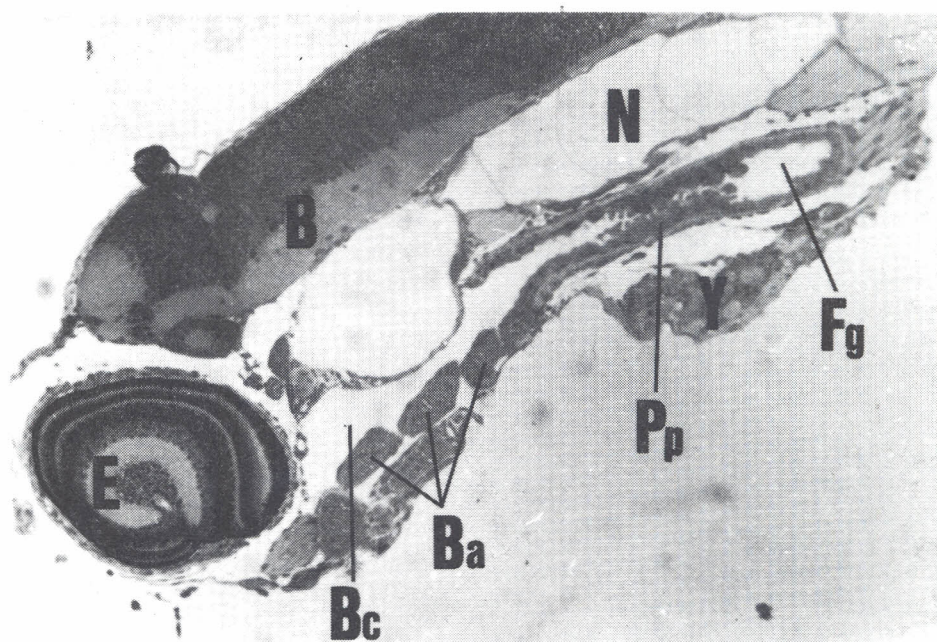


Fig. 1.—Bucco-pharynx of 4.5-day-old larvae ($\times 100$). (B): brain, (Ba): branchial arches, (Bc): buccal cavity, (E): eye, (Fg): foregut, (N): notochord, (Pp): pharyngic port and (Y): remains of the yolk-sac.

Fig. 1.—Zona buco-faríngea de una larva de 4.5 días ($\times 100$). (B): cerebro, (Ba): arcos branquiales, (Bc): cavidad bucal, (E): ojo, (Fg): zona anterior del tubo digestivo, (N): notocorda, (Pp): válvula faríngea e (Y): restos del saco vitelino.

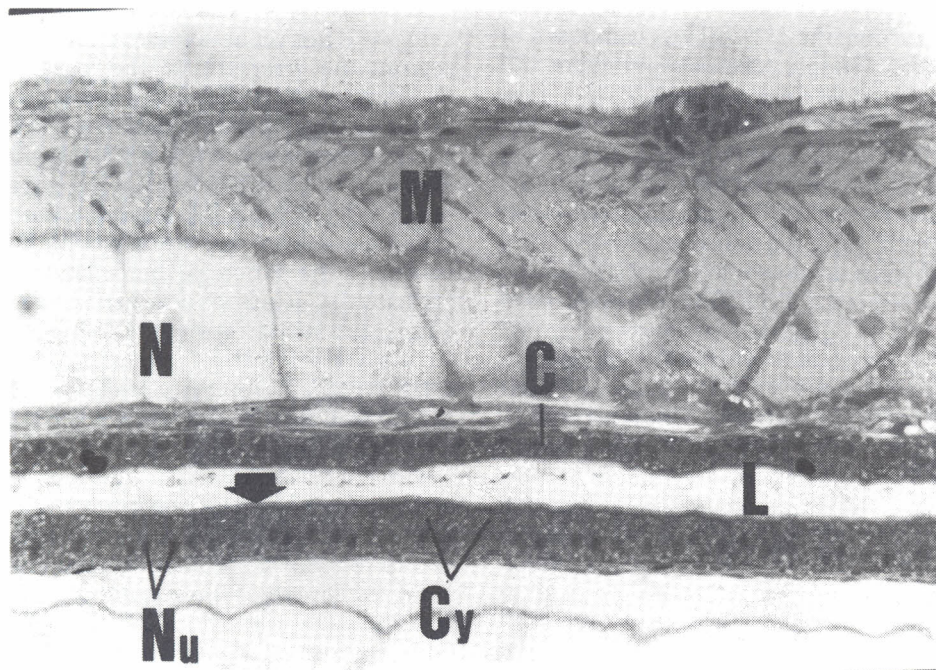


Fig. 2.—Midgut of 4.5-day-old larvae ($\times 250$). (C): ciliated cell, (Cy): epithelial cell cytoplasm, (L): lumen, (M): trunk musculature, (Nu): epithelial cell nucleus and (●): striated border (see previous caption).

Fig. 2.—Zona media del tubo digestivo de una larva de 4.5 días ($\times 250$). (C): célula ciliada, (Cy): citoplasma de una célula epitelial, (L): lumen, (M): musculatura del tronco, (Nu): núcleos de células epiteliales y (●): borde estriado (ver pie de la figura anterior).

cytoplasmic vacuoles (fig. 2). Ciliated cells were already apparent in the posterior half of the gut. The ileocecal valve began to develop. Hindgut cells lacked vacuoles (fig. 3) and were further distinguished from midgut cells by the presence of a band of minute light inclusions below the striated border.

The liver and the pancreas enlarged, increasing the complexity of the internal canalicular system but their histological organization remained unchanged. The most conspicuous features of these glands were still the presence of clear vacuoles in the hepatocytes and of zymogen granules in the acinar cells (fig. 4).

From 5.5 days to 9.5 days, no major structural changes were observed in the digestive organs of sardine larvae. The yolk was not completely absorbed until 7.5 days, indicating that larvae have a mixed nutrition for two days after first-feeding.

One day after food was added, feeding larvae could be distinguished from unfed ones. The feature that first allowed this dis-

tinguishment was the presence of supranuclear vacuoles at the hindgut of feeding larvae (fig. 5). Unfed larvae presented similar characteristics to yolk-sac larvae (see fig. 3). According to Watanabe (1984), these vacuoles correspond to pynocytotic inclusions involved in protein digestion.

At age 7.5 days, other signs of food metabolism were evident: zymogen granules from the pancreatic cells decreased in size and number, concentrating near the centroacinar ducts and hepatocytes developed extensive intracellular "spaces" which reacted positively to the PAS test (fig. 6). This indicated that enzymes were being mobilized for the digestive process and that surplus energy was being stored in the form of glycogen.

Unfed larvae lacked either of these characteristics, their digestive glands keeping the appearance developed during the yolk-sac stage (see fig. 4). However, no signs of degenerative changes were observed in the

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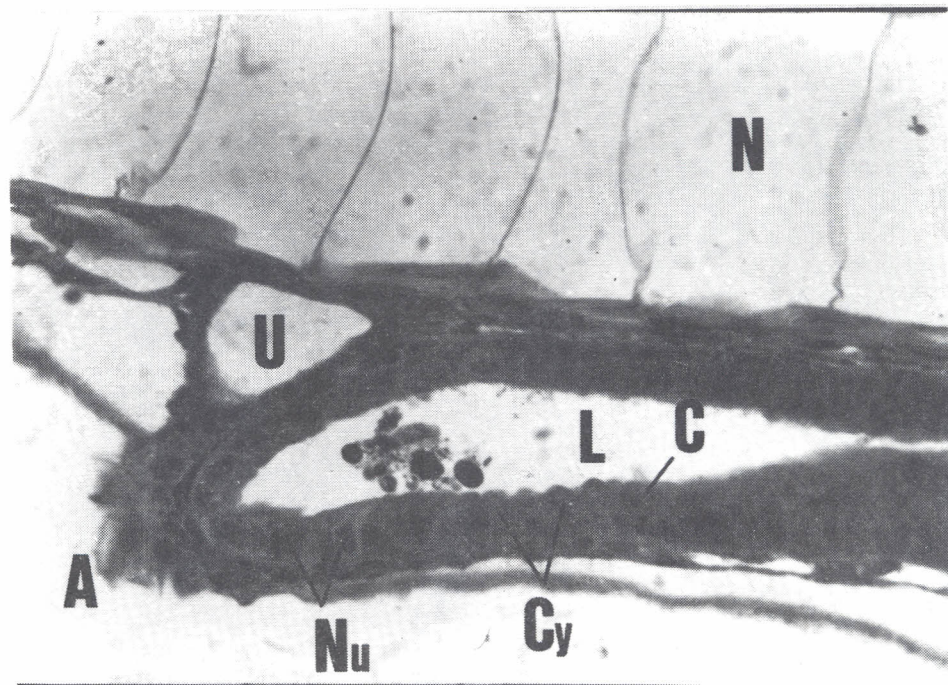


Fig. 3.—Hindgut of 4.5-day-old larvae ($\times 400$). (A): anus and (U): urinary bladder (see previous captions).

Fig. 3.—Zona final del tubo digestivo de una larva de 4.5 días ($\times 400$). (A): ano y (U): vejiga urinaria (ver pies de figuras anteriores).

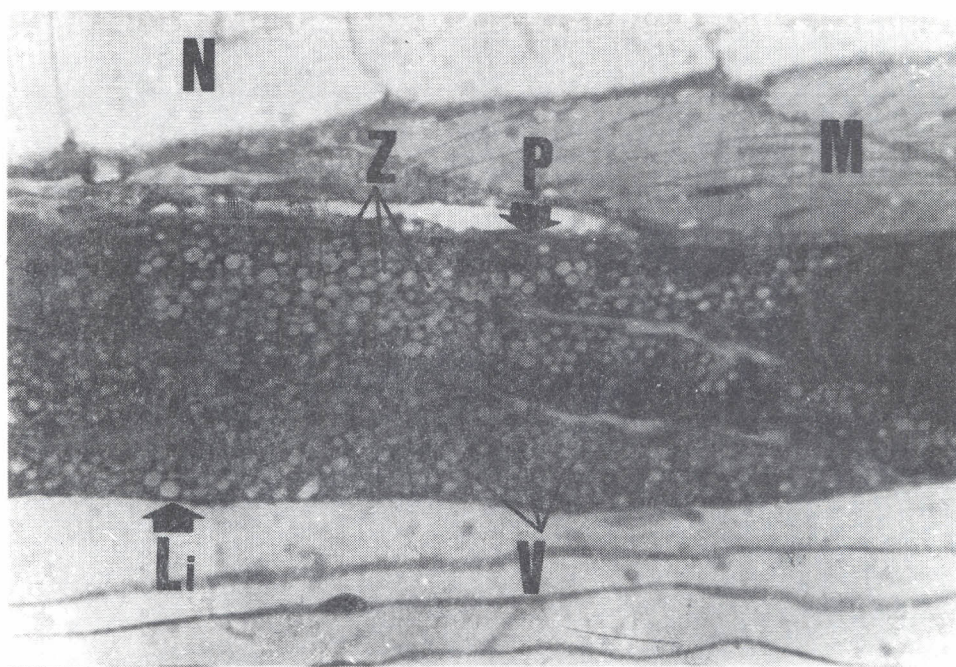


Fig. 4.—Pancreas and liver of 4.5-day-old larvae ($\times 400$). (I): islet of Langerhans, (Li): liver, (P): pancreas, (V): vacuoles and (Z): zymogen granules (see previous captions).

Fig. 4.—Páncreas e hígado de una larva de 4.5 días ($\times 400$). (I): islote de Langerhans, (Li): hígado, (P): páncreas, (V): vacuolas y (Z): gránulos de zimógeno (ver pies de figuras anteriores).

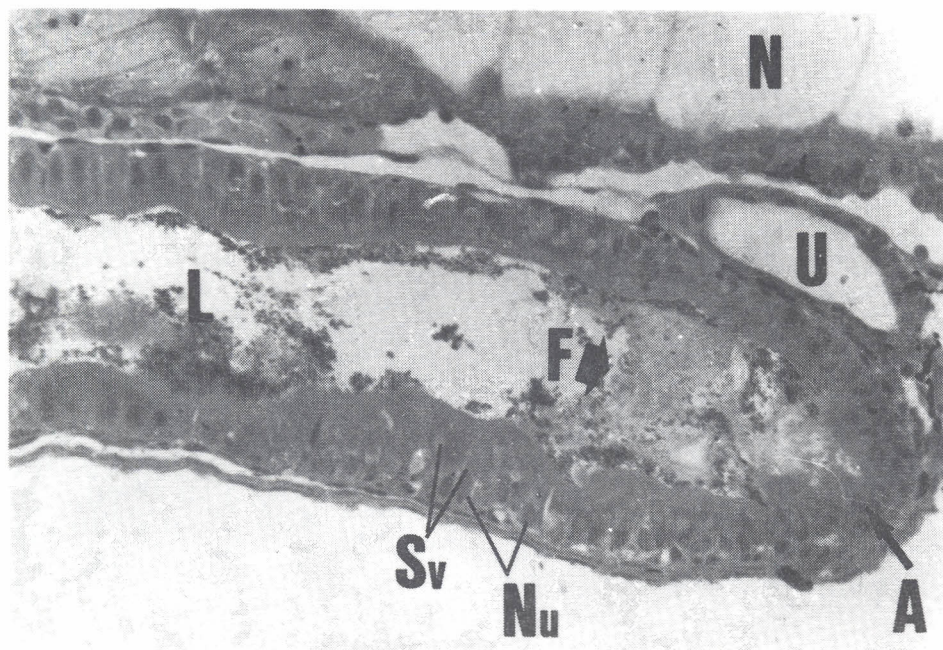


Fig. 5.—Hindgut of 6.5-day-old fed larvae ($\times 250$). (F): food residues and (Sv): supranuclear vacuoles (see previous captions).

Fig. 5.—Zona final del tubo digestivo de una larva de 6.5 días ($\times 250$). (F): residuos alimenticios y (Sv): vacuolas supranucleares (ver pies de figuras anteriores).

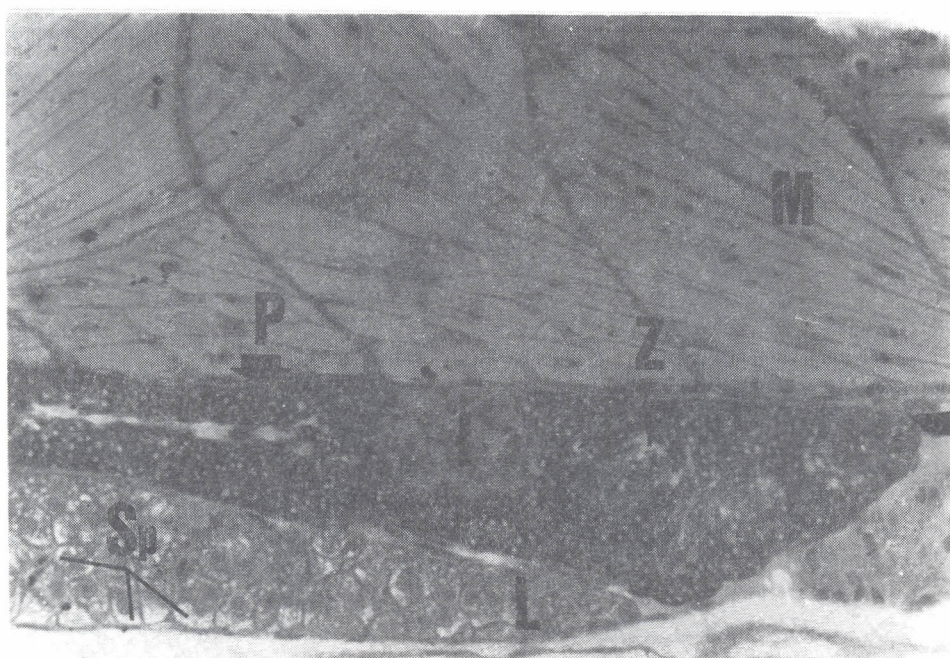


Fig. 6.—Pancreas and liver of 7.5-day-old fed larvae ($\times 250$). (Sp): liver intracellular spaces (see previous captions).

Fig. 6.—Páncreas e hígado de una larva de 7.5 días ($\times 250$). (Sp): espacios intracelulares en el hígado (ver pies de figuras anteriores).

digestive organs or in any other larval organs.

Survival

Hatching success was nearly 85%. On the first day, the overall survival rate for the three larval rearing containers was 66%. The infection of the sardine eggs by the parasitic dinoflagellate, *Ichthyodinium chabellardi* (first reported on sardine eggs from the Iberian coast by Meneses and Ré (1991)), may have been responsible for some of the mortality observed on this day. The analysis of quantitative samples collected at the same time as those used for experimental purposes revealed an infection rate of 55%. We observed a high proportion of infected larvae on the first day of the experiment, but on the following day all sampled larvae were healthy, suggesting that contaminated individuals died in the 24 hours after hatching.

Larval survival rates in the three rearing containers are presented in fig. 7. At first-

feeding stage (day 6), the number of larvae was reduced to 30% of the initial number of larvae in containers *F* and *U*₁. In container *U*₂, all larvae were dead on this day.

Survival was apparently improved when larvae started to ingest food. On the other hand, the number of unfed larvae continued to decrease at a similar rate until all the larvae were dead. Feeding larvae survived for 10 days and the last sampled larvae appeared to be good condition.

Growth

The Laird-Gompertz model provided a very good fit to the observed data for both rearing containers (*U*₁: $r^2 = 0.77$, $n = 65$; *F*: $r^2 = 0.81$, $n = 92$; both $P < 0.001$).

During the yolk-sac period, larvae grew from 3.39 mm (age = 0.5 days) to 5.27 mm (age = 5.5 days (fig. 8)). Thereafter, growth was slower, particularly in non-feeding larvae. Specific growth rates decreased exponentially from $1.3 \text{ mm} \cdot \text{day}^{-1}$ at age 0.5 days to $0.04 \text{ mm} \cdot \text{day}^{-1}$ at age 9.5 days (mean =

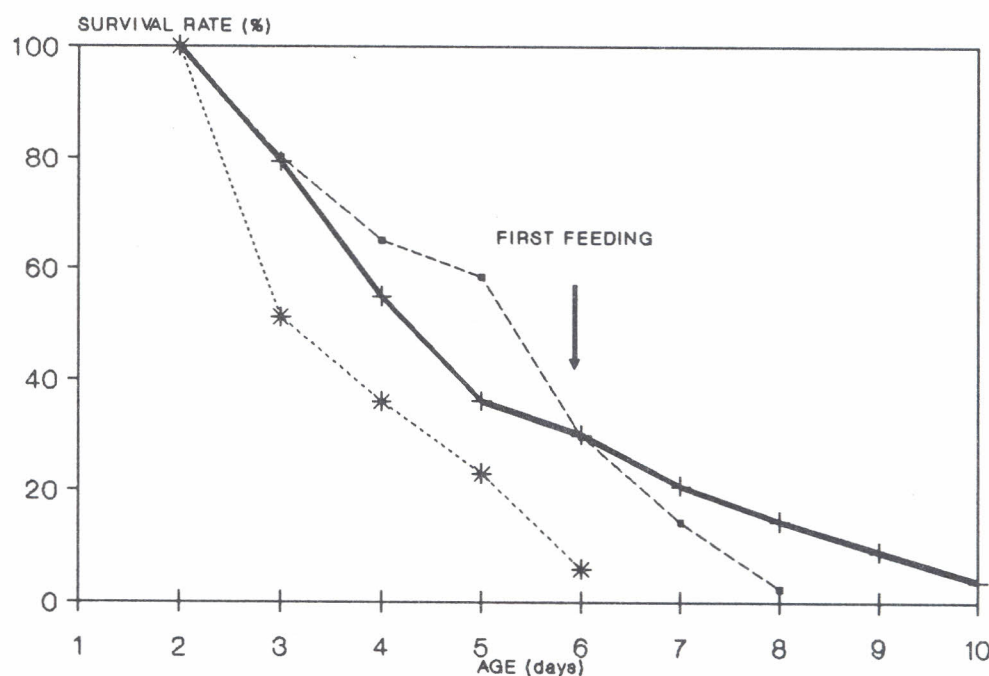


Fig. 7.—Survival of sardine larvae in the three rearing containers, throughout the experimental period. (· · · · ·): container *U*₁ (unfed larvae), (+ —): container *F* (fed larvae) and (* · · · ·): container *U*₂ (unfed larvae).

Fig. 7.—Supervivencia de larvas de sardina en tres recipientes de cultivo, a lo largo del experimento. (· · · · ·): recipiente *U*₁ (larvas sin comida), (+ —): recipiente *F* (larvas con comida) y (* · · · ·): recipiente *U*₂ (larvas sin comida).

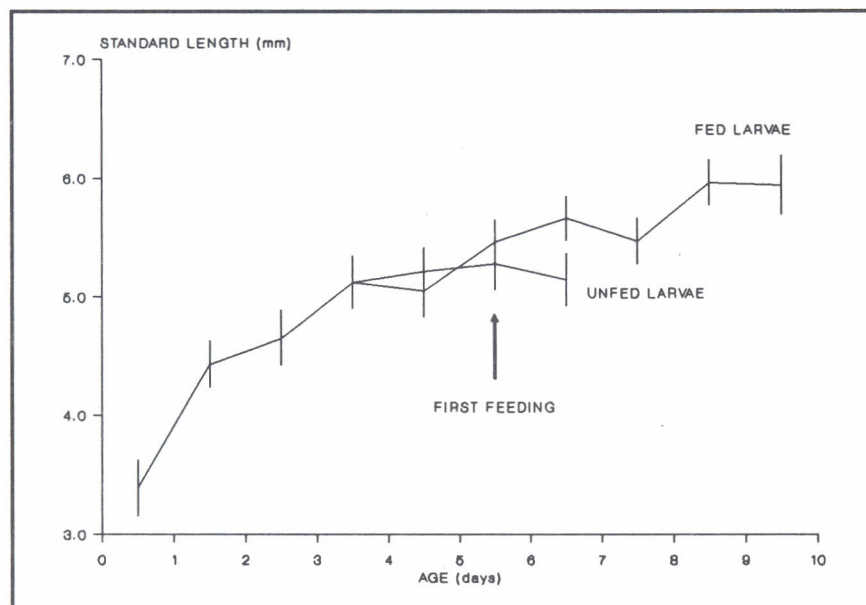


Fig. 8.—Mean values and 95 % confidence limits for larval standard lengths at age in containers *F* (fed larvae) and *U₁* (unfed larvae) throughout the experimental period.

Fig. 8.—Valores medios y límites de confianza del 95 % de las longitudes estándar para cada edad en los tanques *F* (larvas con comida) y *U₁* (larvas con comida) a lo largo del experimento.

$= 0.41 \text{ mm} \cdot \text{day}^{-1}$) for feeding larvae and from $0.51 \text{ mm} \cdot \text{day}^{-1}$ at age 0.5 days to $0.03 \text{ mm} \cdot \text{day}^{-1}$ at age 6.5 days (mean $= 0.13 \text{ mm} \cdot \text{day}^{-1}$) for non-feeding larvae.

DISCUSSION AND CONCLUSIONS

The sequence of development of sardine larvae in laboratory conditions is similar to that described for other Clupeoid larvae (Lasker, 1964; Fukuhara, 1983). The times from hatching to eye pigmentation and absorption of the yolk-sac are comparable to those reported by Miranda, Cal and Iglesias (1990), who also worked with eggs from the Iberian sardine stock. These times are longer than those reported by Blaxter (1969) for the Plymouth sardine at the same temperature, suggesting that the duration of the yolk-sac period depends of the origin of the eggs.

The histological development of sardine larvae digestive system is comparable to other marine fish larvae (Tanaka, 1969; Govoni, 1980). However, the presence of vacuoles in the midgut cells and in the hepatic

cells of yolk-sac larvae contrasts with the findings of other authors. Similar intracellular inclusions have been found only in feeding larvae. In the liver, vacuoles have been identified as glycogen deposits or lipid reserves (O'Connell and Paloma, 1981; Watanabe and Sawada, 1985) and in the midgut as lipidic structures formed by the absorption of nutrients (Lowe and Eckmann, 1988; Segner *et al.*, 1989). Lipid reserves are generally negligible in yolk-sac larvae (Ehrlick, 1974). However, considering the presence of an oil globule in the yolk-sac, and that both hepatic and midgut cells are involved in lipid metabolism, the hypothesis that the observed vacuoles could be lipidic inclusions should not be excluded. We feel that further attention should be paid to this question.

Sardine larvae experienced a high mortality rate. Data on larval survival in laboratory conditions is not consistent; both high (Houde and Palko, 1970; O'Connell and Raymond, 1970) and low (Blaxter, 1968; Yoneda, 1987) survival rates have been reported for yolk-sac larvae.

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and handling stress are some of the factors affecting survival of first larval stages (Blaxter and Hunter, 1982; Li and Mathias, 1982). The design of the experiment, namely the small rearing containers, high initial larval density and handling of recently hatched larvae, might have been responsible for the low survival.

The growth pattern of sardine larvae is comparable to that of other marine fish larvae both in the laboratory (Zweiffel and Lasker, 1976) and at sea (Rayd, 1986). Growth rate is higher immediately after hatching, and a period of minimal growth occurs at the transition to exogenous feeding when nutrition meets only the basic metabolic demands of larvae. The mean daily growth rate of feeding larvae is within the range of daily growth rates of Clupeoid larvae (Blaxter and Hunter, 1982). Apparently, rearing container size did not affect larval growth.

Histological signs of digestive activity were evident one day after food was added. The appearance of pynocytotic vacuoles in the hindgut cells, the accumulation of glycogen in the hepatocytes and the decrease of zymogen granules in the pancreas after first-feeding have been observed in larvae of many species (O'Connell, 1976; Theilacker, 1978; Storch and Juario, 1983; Cousin, Balowet and Baudin-Laurencin, 1986; Oozeki, Ishii and Hirano, 1989; Strussmann and Takashima, 1989; Chantanookookin, Tanawa and Seikal, 1990). Unfed larvae showed none of these characteristics, nor any signs of tissue degeneration, suggesting that larvae did not reach a starvation condition. On the other hand, both survival and growth of non-feeding larvae were impaired soon after food deprivation, which might indicate that although they were not starving, their condition was sub-optimal. However, only a longer experiment with a higher initial number of larvae will enable the confirmation of these results.

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